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Effects of Tannin-Containing Feed on Haemonchous contortus in Sheep and Its Behavioral Implications

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EFFECTS OF TANNIN CONTAINING FEED ON *HAEMONCHOUS*

CONTORTUS IN SHEEP AND ITS BEHAVIORAL

IMPLICATIONS

by

Jessica Juhnke

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ABSTRACT

Effects of Tannin-Containing Feed on *Haemonchous contortus* in Sheep

and Its Behavioral Implications

by

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Herbivores prefer foods that supply required nutrients and avoid those with excess nutrients and plant secondary compounds (PSC). Nevertheless, PSC such as condensed tannins can provide beneficial medicinal effects to herbivores. The objectives of this study were to determine: 1) if parasitized lambs increased preference for a tannin-rich food after they experienced the beneficial antiparasitic effects of tannins relative to parasitized lambs that did not experience such benefits, 2) if preference for the tannin-containing food in the former group decreased when parasite burdens subsided, and 3) if the tannin-enriched food decreased parasitic burdens.

Twenty-two lambs were familiarized with beet pulp and beet pulp + 8% quebracho tannins and choices were given between the two foods (initial preference tests). Subsequently, all animals were dosed with

10,000 L_3 stage larvae of *Haemonchous contortus*. Twenty-two days later, animals were exposed to beet pulp (Control group; n=11) or beet pulp+tannins (Treatment group; n=11) during a span of 24 d. After exposure (during a parasitic infection) animals in both groups were given choices between the two foods. Lastly, animals in both groups received an antiparasitic drench and were again given a choice between both foods (after a parasitic infection). Lambs preferred beet pulp to beet pulp + tannins throughout the study ($P< 0.001$) and no difference in preference for the tannin-rich food was detected between groups during initial preference tests ($P > 0.05$). However, during a parasitic infection, intake of and preference for the tannin-containing food was higher for Treatment lambs than for Control lambs ($P < 0.05$). When parasitic infections were terminated by chemotherapy, differences between groups disappeared ($P > 0.05$). Preference by the Treatment group for the tannin-containing food was lower after than during a parasite infection ($P < 0.05$). In contrast, preference by the Control group did not change during these periods ($P > 0.05$). Lambs in the Treatment group displayed lower FEC than lambs in the Control group (Group x Sampling Date; $P < 0.05$). These results show lambs needed to learn about the beneficial antiparasitic effects of tannins (Treatment) to increase their preference for the tannin-containing food.

(77 pages)

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Jessica Juhnke

For my family

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INTRODUCTION

Internal parasites are one of the greatest disease problems in grazing livestock worldwide (Waller, 2006). Their control through chemotherapy is problematic due to the rise in anthelmintic resistance (Jackson and Miller, 2006). Factors selecting for resistance involve mass treatment, frequent use of the same drug treatment over long periods of time, and under dosing (Geerts et al., 1997). More effective and sustainable programs for combating parasites may be possible if a greater variety of controls are used. For instance, programs that integrate grazing management and biological control, in addition to anthelmintics, are best for controlling parasites and minimizing harmful effects of drugs on the functioning of soil (Strong, 1993; Colwell et al., 2002).

Considerable attention has been given to the anthelmintic properties of phytochemicals, particularly tannins, as an alternative for controlling parasites (Min and Hart, 2003; Ramírez-Restrepo et al., 2005; Mueller-Harvey, 2006). Tannins are a diverse group of polyphenolic compounds, widely distributed among grasses, forbs, and woody plant species, with several benefits to ruminants (Min et al., 2003). Nevertheless, tannins and other phytochemicals at high concentrations cause negative consequences in herbivores (Cheeke and Shull, 1985;

Cheeke, 1988), which lead to significant reductions in animal productivity and welfare.

An alternative to this challenge, which has not been fully explored, is allowing animals to self-select phytochemical containing plants with antiparasitic properties. By allowing each animal to regulate intake of tannins as a function of parasite load, self-medication minimizes chemo-resistance due to both mass treatment and under dosing. Sheep self-medicate with polyethylene glycol as a function of tannin concentrations in their diet (Provenza et al., 2000). Self-medicative behavior in the African great apes appears to fit this pattern and may represent a stable evolutionary strategy for parasite control (Huffman et al., 1998). Evidence for self-medicative behavior against parasitic infections is abundant in primates (Huffman, 2003, 2006; Krief et al., 2004, 2005), and recent studies suggest sheep self-medicate against natural gastrointestinal parasitic infections (Lisonbee et al., 2009; Villalba et al., 2010).

The objectives of this study were 1) to determine whether or not parasitized sheep increased preference for a tannin-containing food after they experienced the beneficial antiparasitic effects of tannins relative to parasitized animals that did not experience the benefits, and 2) if preference for the tannin-containing food decreased when parasite burdens subsided. To do so, a group of lambs (Treatment) was conditioned to consume a tannin-containing food while challenged by an infection of *Haemonchous contortus*, whereas another group of lambs

(Control) was conditioned to consume the same food but without tannins while challenged by the same *H. contortus* infection. Subsequently, all animals received choices between the foods with and without tannins. Finally, parasitic infections were terminated by chemotherapy and choices were offered again to all animals.

Background

Gastrointestinal parasites are one of the largest health problems affecting ruminants today (Jackson and Miller, 2006). Parasites reduce ruminant production and adversely affect their welfare (Athanasiadou et al., 2000). Internal parasites cause chronic ill thrift (Waller, 2006), with major sources to ruminant production including poor growth (Min et al., 2003) appetite loss, diarrhea, anemia (Athanasiadou et al., 2008) and suppression of immunity during reproduction and lactation (Houdijk, 2001). Conventional methods for controlling parasites have been achieved largely through the use of chemotherapeutic drugs; however, increasing nematode resistance to this conventional treatment has spurred interest in new methods of control (Waller, 2006). This has become increasingly relevant as the likelihood of novel veterinary drugs is grim (Geary, 1999). We are now at a point where chemotherapeutic drugs are losing effectiveness while social and consumer concerns about additions of chemicals to food products are gaining traction. Consumers are concerned with possible toxicity of chemical inputs in agriculture and food systems (Waller and Thamsborg, 2004) and they are increasingly concerned about animal care and welfare (Lund, 2006). At this unique point in time scientists from different disciplines are in a position to work together to combine old and new approaches in pursuit of longer term more sustainable solutions to agricultural challenges. One option that we will be investigating is a more integrative approach that combines an understanding of animal physiology and behavior with the environment decrease reliance on chemical inputs in agriculture.

Haemonchous Contortus

Haemonchous contortus are common gastrointestinal parasites in the United States. There are four developmental stages in the life cycle of *H. contortus*. The L_1 and L_2 life stages occur outside the host body in the feces or on the ground if conditions are appropriate, such as warm moist soil and herbage. The L_3 stage is the infective stage of *H. contortus*; the larvae make their way up the grass and wait to be ingested by herbivores. It is within the host body that the final L_4 stage is reached. Adult females will produce eggs while in the abomasum which will be deposited back on the ground where the cycle will start again.

Importance of Tannins in Livestock Production Systems

Plants have nutrients generally viewed as primary compounds, which include carbohydrates, protein, vitamins and minerals. In addition, plants typically produce a host of so-called secondary compounds (phytochemicals) with important roles in the health of plants, soils, herbivores, and humans (Cozier et al., 2006). Novel approaches in ruminant production systems involve allowing animals to select their diets from an array of feed alternatives containing different types and concentrations of primary and secondary compounds in order to facilitate better health and nutrition, while at the same time enhancing sustainability and environmental health (Provenza, 2008; Provenza et al., 2007, 2009).

Condensed tannins (CTs) are a group of plant secondary compounds that have recently come into favor as a possible treatment for GI nematodes (Niezen et al., 1995; Min et al., 2003; Min and Hart, 2003). Tannins are polyphenolic compounds, stored in the vacuoles of plant cells, and used in part as plant defenses against herbivores. At high concentrations, tannins have negative consequences such as lesions in gastrointestinal mucosa (Dawson et al., 1999) or dramatic and rapid reductions in food intake (Provenza et al., 1990). Even with their potential harmful side effects in too high amounts, CT's have important medicinal properties (Waghorn, 1990).

Recent studies demonstrate multiple positive effects of tannins on livestock production. An increase in weight gain in lambs has been attributed to drenches with sulla, a CT-containing forage (Niezen et al., 1995, 1998). Tannins improve nutrition of ruminants by binding to protein in the pH neutral (pH 6.0-7.0) rumen and making it unavailable for rumen microbial digestion and absorption until it reaches the more acidic abomasum (pH 3.5) and small intestine where dissociation occurs resulting in improved nutritional status (Barry et al., 2001; Min and Hart., 2003). This bonding occurs in the form of hydrogen/hydrophobic complexes (Hagerman and Butler, 1981; Haslem, 1989). This high quality bypass-protein provides several positive effects to the host including enhanced immune response and increased resistance to infection (Niezen et al., 2002; Min et al., 2003, 2004). Condensed tannins also increase wool growth of parasitized and non-parasitized lambs (Niezen et al., 1998; Luque et. al., 2000), and they may play a role in the reproductive health by increasing ovulation rates and fecundity (Luque et al., 2000). Tannins in the diet also reduce methane emission in ruminants (Woodward et al., 2004).

Considerable attention has been given recently to the anthelmintic properties of tannins (Jackson and Miller, 2006). Condensed tannins may directly affect parasites by interfering with larval development and hatching (Molan et al., 2002) or by causing autolysis (Schultz, 1989). Possibly the most common effect is reduced female fecundity after short-term exposure to CT (Min et al., 2004).

Ability of Animals to Forage in Ways Beneficial to Their Internal Status - Choice and Diversity in Forages

Livestock are intimately tied to the plants and landscapes they feed upon. Plant-herbivore interactions such as energy and nutrient cycling as well as growth rate and organic matter decomposition in soils generate direct and indirect feedback loops on plant community structure (Augustine and McNaughton, 1998). Therefore, management policies that encourage variety in seeding of the land as well as animals trained to indulge in that variety are likely to maintain greater soil, plant, and animal health, and biodiversity. Encouraging biodiversity promotes greater health and adaptability of the organisms that subsist on the land, while imparting a resiliency not seen in monocultures (Provenza et al., 2003). If we are able to achieve this biodiversity on our landscapes, what can we do with it to affect ruminants? One option is to tap into the ability of animal's to choose. But where does choice come from? Is it purely the result of chance acting upon a system? While this may be true in rare instances, mounting evidence suggests choice comes from experience and an animal's ability to discriminate amongst foods based on postingestive feedback from nutrients and plant secondary compounds (Provenza et al., 2003).

Individuals operate on the environment, i.e., they behave, and their behavior has consequences that in turn affect subsequent behavior (Skinner, 1981). In the case of foraging, behavior (preference) by consequences is manifest as the interrelationship between a food's flavor and its postingestive feedback (consequences) (Provenza, 1995; Provenza and Villalba, 2006). The senses of smell, taste, and sight enable animals to discriminate among foods and provide pleasant sensations liking for a food’s flavor associated with eating. Postingestive feedback calibrates sensory experiences like or dislike in accord with a food's utility to the body. For instance, if a particular food provides negative effects to the consumer’s body (e.g., excess toxins, excess nutrients); preference for that particular food will decrease. In contrast, if a food provides chemicals the individual needs at a particular point in time (e.g., nutrient, medicine), preference for that food will increase. Sheep prefer the flavors of foods associated with the delivery of nutrients to their rumens, and they balance the ratio of energy:protein in their diets (Villalba and Provenza, 1999; Provenza and Villalba, 2006).

Besides balancing nutrient intake and avoiding toxins, herbivores are faced with other environmental challenges such as avoiding disease or restoring health. If the concept of behavior by consequences holds, then sick animals should consume substances that restore their health. In support of this prediction, sheep learn to consume medicinal substances to attenuate negative internal states induced by tannins (Villalba and Provenza, 2001). When eating high-tannin foods, they also prefer to forage in locations where a medicine (PEG), which attenuates the negative postingestive effects of tannins, is present (Villalba and Provenza, 2002). Sheep fed acid-producing substrates such as grains subsequently ingest foods and solutions that contain sodium bicarbonate, which attenuates acidosis (Phy and Provenza, 1998). Sheep also learn to selectively ingest three medicines sodium bentonite, polyethylene glycol, dicalcium phosphate that lead to recovery from illness due to eating too high amounts of grain, tannins, and oxalic acid, respectively (Villalba et al., 2006b). Thus, self-medication is a specific adaptive change in behavior in response to sickness. It is characterized by a change in behavior induced by the environment that improves the odds of survival and reproduction (Singer et al., 2009).

But what about plant secondary compounds? They too cause postingestive feedbacks that stem from the nutritional and toxicological characteristics of the diet and the physiological state of the animal (Provenza et al., 2003). Given the anthelmintic properties of tannins described in the previous section, can livestock learn to self-medicate with some of these antiparasitic, but potentially toxic substances? Again, if the concept of behavior by consequences holds, then a parasitized animal should be able to increase preference for a plant secondary compound, if the compound reduces parasitism and restores health.

Wild chimpanzees suffering from parasite-related diseases consume the bitter pith of the plant *Vernonia amygdalina* (Huffman and Seifu, 1989), which contains sesquiterpene lactones and steroid glucosides with antiparasitic activity at the doses consumed by the animals (Koshimizu et al., 1994). Other plants selected by chimpanzees have medicinal effects at the doses consumed: Limonoids in *Trichilia rubescens* have antimalarial activity (Krief et al., 2004); polyacetylenic (Thiarubines) compounds in *Aspilia* spp. confer antiparasitic and antibiotic properties, and methoxypsoralen in *Ficus exasperata* is a strong antibiotic (Rodriguez and Wrangham, 1993). Thus, pioneering studies of apes suggest self-medication with PSC by parasitized animals is possible. Still the question remains, is it possible for livestock? Emerging evidence such as parasitized sheep self-medicating with tannin-containing foods (Lisonbee et al., 2009; Villalba et al., 2010) suggests this may be possible.

What Can Secondary Compounds Mean for the Rest of Us?

In the not so distant past we sought to harvest and encourage only those species which were abundant, palatable and easily cultivated (Etkin, 1994). In doing so we narrowed a vast array of plants to a few which generally fail to meet the full nutritional needs but limit over-ingestion of toxins (Johns, 1994). Embracing these less chemically diverse plants was an easy option during the last century while we reaped the benefits of the Green Revolution; with plentiful fossil fuels being allocated to almost every facet of the agricultural business. Fossil fuels are a component of everything from fertilizer, pesticides and herbicides, pharmaceuticals for the animals and the fuel to run equipment. With rising fuel costs and the likelihood of peak oil looming, along with decreased chemical effectiveness and declining discovery of new pharmaceuticals, new strategies are needed to adapt agricultural production to these new realities. Use of plant secondary compounds and their health benefits, while not a solution in and of themselves may prove to be an important part of the solution.

Objectives, Hypothesis and Predictions

I hypothesize parasitized herbivores will learn to associate the benefits of consuming foods containing plant secondary compounds with anti-parasitic properties. I predict parasitized sheep conditioned to experience the beneficial effects of tannins on parasitic loads will show a greater preference for tannin-containing foods than parasitized sheep which did not experienced such beneficial effects. I further predict this greater preference for tannin-containing foods will decrease when parasitic loads are terminated due to chemotherapy as in this new context the beneficial function of tannins is reduced. Finally, I predict animals consuming tannin-containing foods will have reduced fecal egg counts (FEC), an indirect measurement of parasitic burdens and improved clinical indicators of health such as hematocrit.

To test these hypotheses and predictions, I designed an experiment to assess the ability of parasitized lambs to form preferences for phytochemical-containing foods with antiparasitic properties. I familiarized non-parasitized lambs to the same food (beet pulp) with (medicinal) or without (non-medicinal) tannins. Subsequently, I dosed all lambs with 10,000 infective larvae of *Haemonchous contortus* and exposed half of the animals to the antiparasitic food (beet pulp+tannins) and the other half to the non-medicinal food (beet pulp). After conditioning, all animals were offered choices between beet pulp+tannins and beet pulp. Finally, all animals were drenched with antiparasitic drugs such that their parasitic burdens were reduced, and all animals were offered again choices between beet pulp+tannins and beet pulp.

MATERIALS AND METHODS

The study was conducted at the Green Canyon Ecology Center, located at Utah State University in Logan according to procedures approved by the Utah State University Institutional Animal Care and Use Committee (Approval #1413). During the study, 22 commercial Finn-Columbia-Polypay-Suffolk crossbred lambs (5 mo of age) with an average BW of 48 ± 6 kg. were individually penned outdoors, under a protective roof in individual, adjacent pens measuring 2.4 x 3.6 m. Throughout the study, lambs had free access to fresh water and trace mineral salt blocks. Two weeks before the start of the experiment, sheep were drenched with the anthelmintics pyrantel pamoate 25 mg/Kg and albendazole (11.36%) 1 ml/10 lb. 7.5 mg/Kg BW. Ten days later, fecal samples were taken at 0800 from the rectum, stored in an ice chest and analyzed for fecal egg counts (FEC) during the same day to ensure animals had very low to nil parasitic burden before the start of the study.

Experimental Approach

Lambs were familiarized with a low crude protein food (beet pulp) and the same food containing 8% quebracho tannins (beet pulp+tannins). The low-CP diet was used to maximize the antiparasitic effects of quebracho tannin. Condensed tannins form strong complexes with proteins (Makkar et al., 1987), which can potentially neutralize the effects of condensed tannins. Quebracho tannin is less effective at combating parasites when diets containing high-ptotein (22% CP) as opposed to low-protein (9.7% CP) diets (Butter et al., 2000).

I first established baseline preferences by offering lambs a choice between beet pulp and beet pulp + tannins (Phase 1). Next, lambs were dosed orally with L_3 stage *H. contortus* larva (Phase 2). Twenty-two days after larval dosage lambs were then divided into 2 groups: parasitized lambs in the Treatment group were offered the medicinal food beet pulp + tannins, whereas parasitized lambs in the Control group were offered only beet pulp (Phase 3). After this exposure, I once again determined preference by offering all animals a choice between beet pulp and beet pulp + tannins. Finally, animals were drenched with anthelmintics (Phase 5), and given a choice between the two foods (Phase 6).

Sampling Feces and Blood

Samples of feces and blood where taken at regular intervals throughout the trial corresponding mostly with choice/preference tests. We collected blood to determine PCV on September 24, October 7, 16, 23, and 30, and November 13, 2009. Additional vials of blood (ethylenediaminetetraacetic acid-anticoagulated) were collected on November 13, 2009 for Hemavet analysis of red cell parameters. We collected feces to determine FEC on September 11, 16, and 24, October 2, 7, 16, 23, and 30, and November 13, 2009.

Phase 1: Exposure to Test Foods and Initial Preference Tests

To familiarize lambs with the test foods, all lambs were offered ground beet pulp (1-2 mm particle size) ad libitum from 0900 to 1600 for 5 d (August 20 to August 24, 2009). Refusals were collected and food intake recorded. No other food was offered until the next day. For the next 5 d (August 25 to August 29, 2009), all lambs were fed ground beet pulp with 8% quebracho tannin (Tannin Corporation, Peabody, MA) (as-fed basis). Concentrations of condensed tannins in this range were reported to reduce FEC in sheep (Min and Hart, 2003).

After the 5 day exposures to the test foods, all lambs were offered a choice between beet pulp and beet pulp:tannins from 0900 to 1600 for 2 consecutive days August 30 and 31, 2009, Test foods (≈ 1000 g) were offered simultaneously in separate plastic containers that fit tightly into a wooden food box attached to each pen. The placement (left vs. right) of specific foods was random across pens and days. At 1600, refusals were collected and intake was calculated. No other food was offered until the following day.

Phase 2: Infection

All animals were dosed orally with a single dose of 10,000 L_3 *H. contortus* larva on September 7, 2009. Feces from a donor lamb infected with *H. contortus* were cultured for 10 days at 20 °C and infected larvae were harvested by a standard Baermann procedure. The larvae were stored at 4 °C and used within 2 weeks. Fecal samples were taken at 0800 from the rectum of each animal, stored in an ice chest and analyzed for fecal egg counts (FEC) during the same day. The number of eggs per gram of fresh feces (epg) was determined using the McMaster egg counting procedure for quantifying nematode eggs. We performed FEC 3 days after infection on September 11, 2009 and subsequently on September 16 and 24, 2009. By September 24, 2009 adequate infection of at least 1,000 epg/g, determined by average of epg/g, was reached. Lambs were stratified according to FEC obtained during that day, and pairs of lambs were randomly assigned to the 2 groups (11 lambs/group). Thus, differences between groups due to different FEC were balanced.

Phase 3: Conditioning

In this phase, I conditioned animals in the Treatment group to beet pulp + tannins, and animals in the Control group to beet pulp. I then measured the effect of tannins on FEC and packed cell volume (PCV).

From 0900 to 1600, lambs in the Treatment group were offered the tannin-containing food (8% quebracho, 92% beet pulp), whereas lambs in the control group were offered only beet pulp. At 1600, refusals were collected and intake was calculated. No other food was offered until the following day. Conditioning occurred for 24 d, from September 29 to October 22, 2009.

I performed FEC on all animals on October 2, 7, 16, and 23, 2009 until FEC's stabilized. Jugular blood samples were collected to quantify packed cell volume (PCV).

At 0900 h, all lambs were offered a choice between beet pulp and the beet pulp:tannin food as described for Phase 1. Food refusals were collected at 1000 h, weighed and offered again until 1600, when refusals were collected and weighed to calculate intake of both foods. Thus, we determined intake 1 h and 7 h after offering both test foods. No other food was offered until the following day. Testing was carried out for 5 d during conditioning: October 2, 7, 8, and 16, 17, 2009.

Phase 4: Testing During a Parasitic Infection

After parasite FEC peaked and conditioning (Phase 3) ended, testing occurred during 7 d, from October 23 to October 30, 2009; data was not collected October 27, 2009 due to inclement weather. Testing was conducted as described in Testing During Conditioning. Thus, intake of each food was calculated 1 h and 7 h after offering both foods.

We performed FEC and PCV on all animals on the last day of testing (October 30, 2009) during a parasitic infection. In addition, blood samples (ethylenediaminetetraacetic acid-anticoagulated) were obtained from the jugular vein of each animal and analyzed on the day of submission (within 12 h) on an Hemavet HV950FS (Drew Scientific, Oxford, UK) hematology analyzer.

Phase 5: Treatment with Anthelmintics

On November 5-6, 2009, all lambs were drenched with pyrantel pamoate 25 mg/Kg and albendazole (11.36%) 1 ml/10 lb. 7.5 mg/Kg BW.

We performed FEC and PCV on all animals on November 13, 2009 to assure animals had low to nil parasitic burdens, as determined by FEC.

Phase 6: Testing Without a Parasitic Infection

On November 13, all animals were given access to both foods and consumption was recorded after 1 hand then again after 7 h, as previously in Testing During Conditioining. Testing occurred during 7 d.

Statistical Analyses

Food intake (as fed basis), preference for tannin-containing food (intake of tannin-containing feed/total intake) x 100], fecal egg counts (epg), packed cell volume (PCV) and blood parameters from the Hemavet were analyzed as a split-plot design with lambs (random factor) nested within group. Group (Treatment, Control) and food (tannin-containing feed; beet pulp) were the between animal factor and day was the repeated measure in the analyses (fixed factors).

To determine whether intake of test foods and preference for the tannin-containing food changed during a parasitic infection, intake was analyzed as previously described but day (7 d) and period (1= Phase 4

"Testing during a Parasitic Infection"; 2= Phase 6: "Testing without a Parasitic Infection") were the repeated measures in the split plot.

Analyses were computed using a mixed model (MIXED procedure; SAS Inst., Inc. Cary, NC; Version 9.1 for Windows). The model diagnostics included testing for a normal distribution of the error residuals and homogeneity of variance. Means were analyzed using pairwise differences (DIFF) of least squares means (LSMEANS).

Statistical significance was set at $P \leq 0.05$, however on occasion we decided it was prudent to move significance up to $P \leq 0.15$.

RESULTS

Initial Exposure to the Test Feeds

During the initial 5 d of exposure to each of the test foods, lambs in the Control group ate more than the Treatment group of beet pulp (881 vs. 720 g; SEM = 39 g; $P < 0.05$) and beet pulp+tannin (1173 vs. 1004 g; SEM = 29 g; $P < 0.001$).

Initial Preference Tests

Lambs in both groups preferred beet pulp to tannins+beet pulp on both test days ($P < 0.001$; Fig. 1). Their preference for tannin-containing food was higher on day 1 than on day 2 ($P < 0.001$). No differences between groups were detected for intake of beet pulp or beet pulp+tannins (Group effect; $P = 0.22$, Group X Feed; $P = 0.20$; Group X Feed X Day; $P = 0.29$), or for preference for beet pulp+tannins (Group effect $P = 0.65$; Group X Day; $P = 0.19$).

Intake of Test Foods During Conditioning

During the 24 d of conditioning, lambs in the Control group ate 1298 g of beet pulp, while lambs in the Treatment group ate 862 g of beet pulp+tannins (SEM = 15 g; $P < 0.001$).

A

B

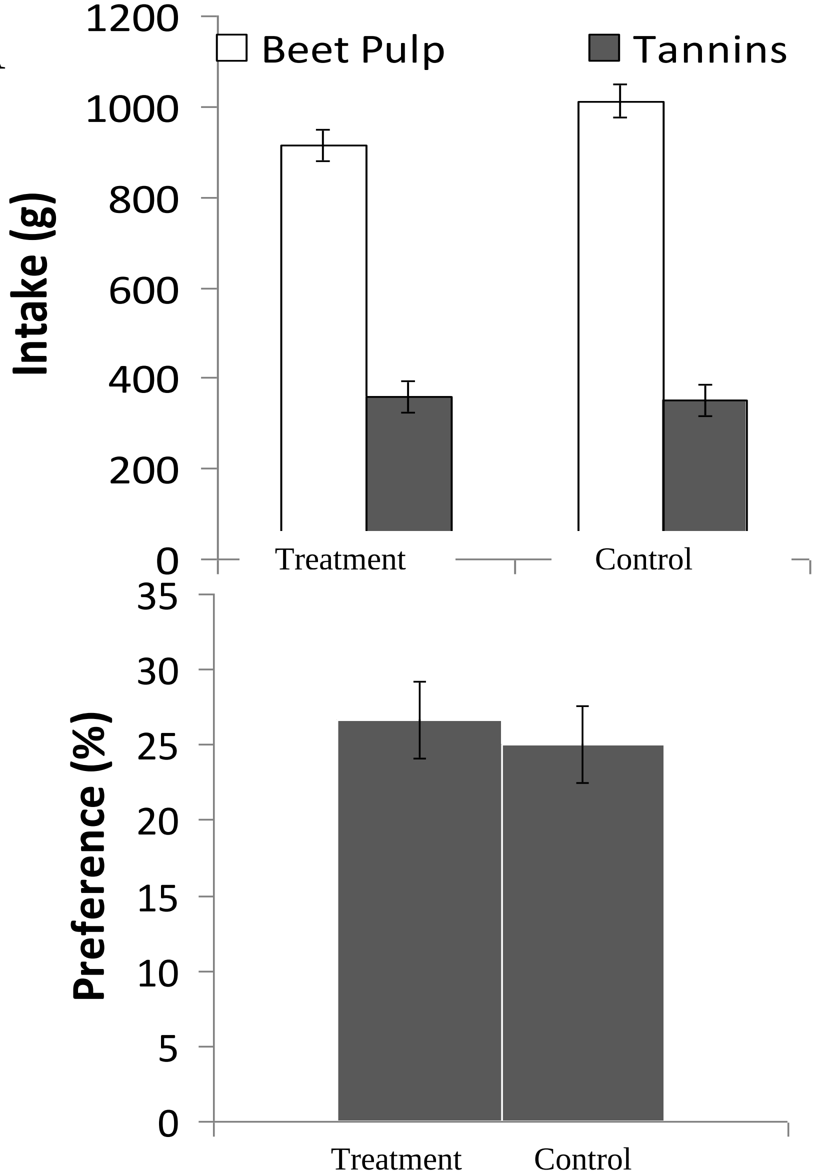

Fig. 1. Initial intake of beet pulp and tannin-containing beet pulp (A) and preference for tannin-containing beet pulp (B) during preference tests by two groups of lambs before being conditioned (Treatment) or not (Control) to the beneficial effects of tannins. Bars represent SEM.

Intake and Preference for Test Foods During a Parasitic Infection

During the first hour, lambs ate more beet pulp than tannin+beet pulp ($P < 0.001$). Groups ate different amounts of the test foods across days (Group X Feed X Day; $P < 0.001$; Fig. 2). Lambs in the Treatment group ate more tannin-containing food than lambs in the Control group on Oct 16 ($P < 0.05$), Oct 23-24 ($P = 0.10$), and Oct 25 ($P < 0.05$). Oct 16-25 corresponded to a time when lambs were experiencing the highest parasite infection, as determined FEC, and when differences in FEC were also detected between groups (see below).

During the daily 7-h preference tests, lambs also ate more beet pulp than tannin+beet pulp ($P < 0.001$), but lambs in the Treatment group ate more tannin-containing food and less beet pulp than lambs in the Control group (Group x Feed; $P = 0.02$; Figure 2). Differences occurred when lambs had the highest parasite infection as determined by FEC from Oct 16-25 (Group X Feed X Day; $P < 0.001$).

Preference for tannin-containing food was higher for lambs that experienced the beneficial effects of tannins while parasitized (Treatment) than for lambs that did not (Control) (first hour of presentation of foods; Group effect $P = 0.02$; 7-hour preference tests: Group effect $P = 0.009$: Fig. 3).

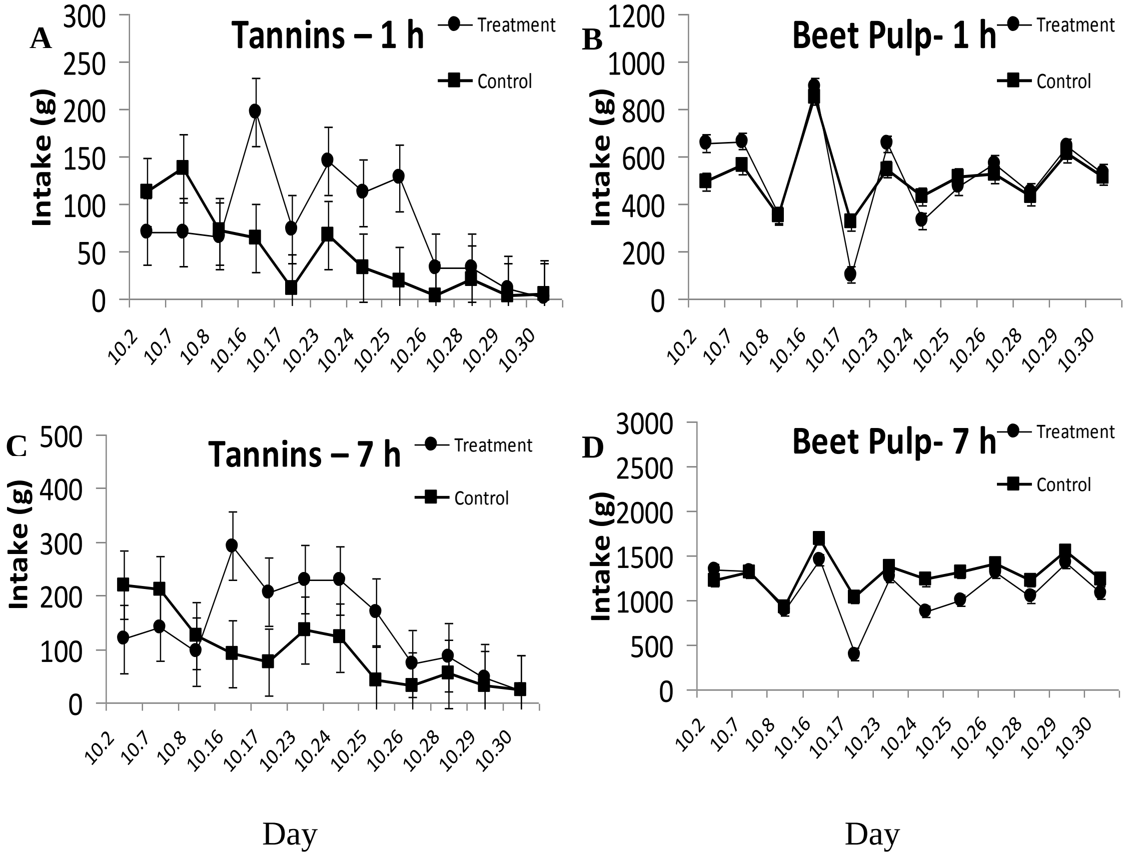

Fig. 2. Intake of tannin-containing beet pulp and beet pulp during the first hour of preference tests (A, B) and after 7 h of preference tests (C, D) by two parasitized groups of lambs. Treatment lambs conditioned to experience the beneficial effects of tannins reducing parasitic loads. In contrast Control lambs did not experience the beneficial effects of tannins during conditioning. Bars represent SEM.

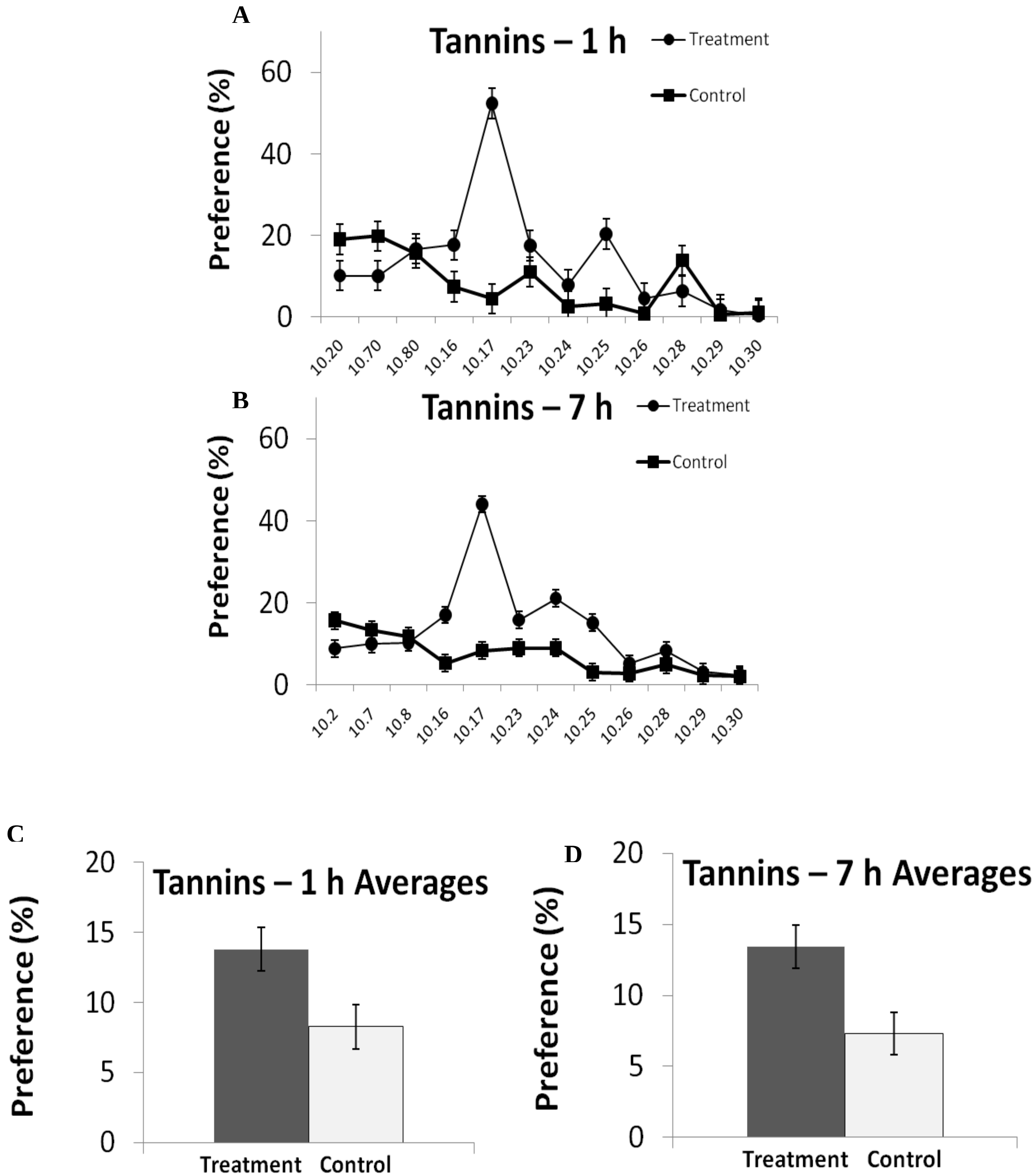
A
Tannins – 1 h
Treatment
Control
Preference (%)
60
40
20
0
10.20
10.70
10.80
10.16
10.17
10.23
10.24
10.25
10.26
10.28
10.29
10.30
B
Tannins – 7 h
Treatment
Control
Preference (%)
60
40
20
0
10.2
10.7
10.8
10.16
10.17
10.23
10.24
10.25
10.26
10.28
10.29
10.30
C
Tannins – 1 h Averages
Preference (%)
20
15
10
5
0
Treatment
Control
D
Tannins – 7 h Averages
Preference (%)
20
15
10
5
0
Treatment
Control

Fig. 3. Preference for tannin-containing beet pulp during preference tests by two parasitized groups of lambs (A, B, C, and D). Treatment lambs were conditioned to experience the beneficial effects of tannins at reducing parasitic loads. In contrast Control lambs did not experience the beneficial effects of tannins during conditioning. Bars represent SEM.

Intake and Preference for Test Foods After a Parasitic Infection

When parasitic infections were terminated by chemotherapy, lambs in both groups continued to prefer beet pulp over beet pulp+tannins ($P < 0.001$). Lambs in the Treatment group continued to consume more tannin-containing food than Controls for the first hour of presentation of foods (Group X Feed; $P = 0.07$, Group X Feed X Day; $P < 0.001$; Figure 4). However, differences between groups disappeared for the entire 7-h preference test (Group X Feed; $P = 0.27$, Group X Feed X Day; $P < 0.60$: Figure 4). No differences in preference for the tannin-containing food were observed between groups for the first hour (Group effect $P = 0.22$) or the entire 7-hour preference test (Group effect $P = 0.41$) (Fig. 4).

Preference for the tannin-containing food during the 7-h preference test was lower for the Treatment group after a parasitic infection (7.0%; Phase 6), than during a parasitic infection (10.2%; Phase 4) (SEM = 1.4%; $P < 0.05$). In contrast, preference did not change for the Control group after a parasitic infection (5.4%; Phase 6), relative to when a parasitic infection was present (4.7%; Phase 4) (SEM = 1.4%; $P > 0.05$), which caused a Group X Phase interaction ($P < 0.05$). No other differences were detected between phases ($P > 0.10$), except lambs in the Control group ate more beet pulp for the first hour of presentation of feeds offered in Phase 6 (617 g) compared with Phase 4 (511 g; SEM = 27 g) (Group X Feed X Phase; $P = 0.10$) .

Fecal Egg Counts

Groups differed in FEC across time (Group x Sampling Date; $P < 0.05$). Lambs in the Treatment group displayed lower FEC than lambs in the Control group on October 2 ($P<0.001$), 16 ($P = 0.11$), and 23 ($P= 0.06$) (Fig. 5).

Packed Cell Volume

No differences in PCV were detected between groups of lambs (Group x Sapling Date; $P > 0.05$). There was a sampling date effect ($P < 0.001$). Values increased toward the end of the study (Nov 13; Figure 5), consistent with a decrease in parasitic burdens due to chemotherapy (performed on Nov. 5-6), and low FEC values (Fig. 5).

Red Cell Parameters

No differences between groups were detected in the red cell parameters for blood collected on the last day of testing during a parasitic infection ($P > 0.05$; Table 1). Red blood cell distribution width ($P < 0.09$) and mean cell volume ($P = 0.12$) tended to be higher for the Control than for the Treatment group (Table 1).

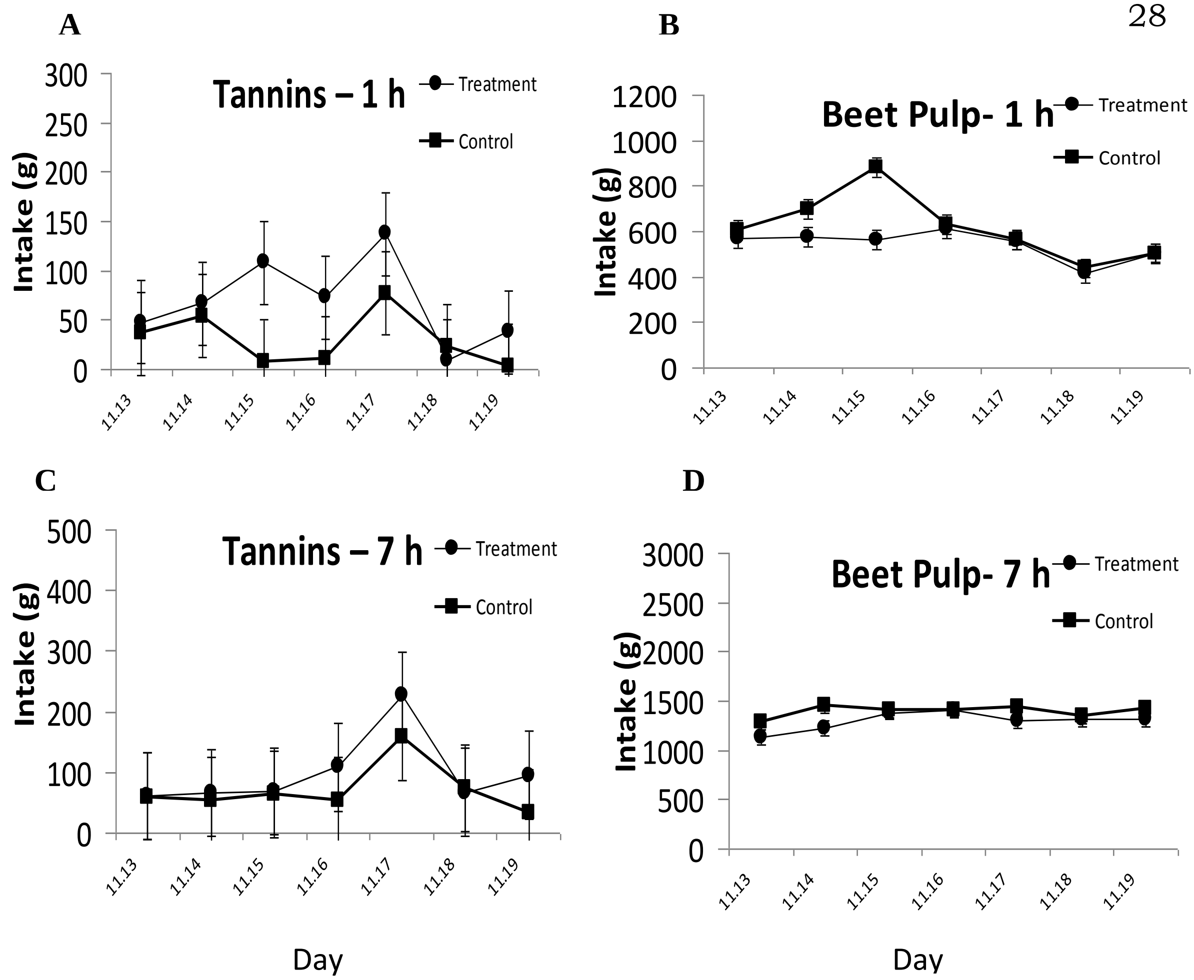

Fig. 4. Intake of tannin-containing beet pulp and beet pulp during the first hour (A, B) and after 7 h (C, D) of preference tests by two groups of lambs after receiving anthelmintics. The Treatment group was conditioned to experience the beneficial effects of tannins at reducing parasitic loads. In contrast, the Control group did not experience the beneficial effects of tannins during conditioning. Bars represent SEM.

A

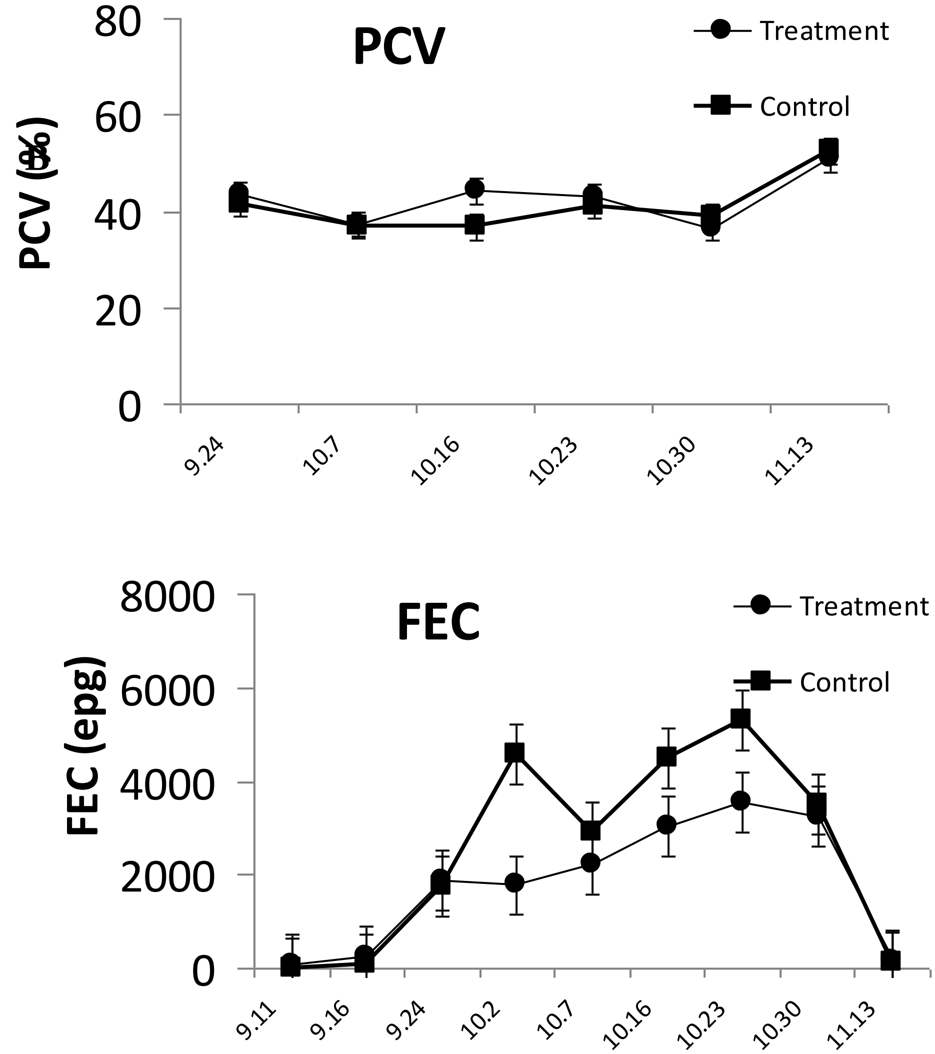

Fig. 5. Packed cell volume (PCV) (A) and fecal egg counts (B) for two parasitized groups of lambs during the study. Treatment lambs were conditioned to experience the beneficial effects of tannins at reducing parasitic loads. In contrast, Control lambs did not experience the beneficial effects of tannins during conditioning. Bars represent SEM.

Table 1. Red cell parameters in two groups of lambs after offering choices between beet pulp and tannin-beet pulp foods. Treatment lambs were conditioned to experience the beneficial effects of tannins at reducing parasitic loads. In contrast, Control lambs did not experience the beneficial effects of tannins during conditioning.

Parameter	Group: Treatment	Group: Control	SEM	*P*
Red Blood cells (M/µl)	10.28	10.23	0.577	0.95
Hemoglobin level (g/dl)	8.0	8.2	0.45	0.82
Hematocrit (%)	29.05	30.30	1.47	0.56
Mean Cell Volume (fl)	28.3	29.9	0.68	0.12
Mean Cell Hemoglobin (pg)	7.8	8.1	0.21	0.38
Mean Cell Hemoglobin Concentration (g/dl)	27.5	26.9	0.35	0.27
Red Cell Distribution Width (%)	24.1	26.2	0.84	0.09

DISCUSSION

Initial Choice and Intake

As expected, lambs in both groups consumed more beet pulp than tannin + beet pulp mixture. The tannin-containing food was of lower nutritional value due to dilution with tannins. Additionally, tannins can have negative effects on herbivores at high concentrations (Mehansho et al., 1987; Provenza et al., 1990). Lambs consumed more of the tannin food on the second day of testing, which could be attributed to a greater familiarization with the food or to a lower preference for beet pulp due to a higher consumption of this food on the previous day. Ruminants reduce intake of foods consumed too frequently or in excess (Provenza, 1996). Initial preference tests also showed no differences in food intake or preference for the tannin-containing food between groups. Thus, I attribute subsequent differences in preference to the treatments applied to both groups of animals during conditioning

Choice During and After Parasitic Infection

I hypothesized lambs infected with endoparasites would learn the benefits of consuming the beet pulp + tannin food with antiparasitic properties as a consequence of experiencing relief from infection after consuming the medicinal food. Consistent with this, consumption of tannins and preference for the tannin-containing food increased appreciably in the parasitized group of lambs that experienced the medicinal effects of tannins during conditioning relative to the parasitized group that did not. Moreover, increased preference for the tannin-containing food occurred on days when infection, based on FEC, was highest (Oct 16-25). These results suggest lambs needed to learn from experience about the beneficial antiparasitic effects of consuming tannins.

Results from this study are consistent with previous findings suggesting parasitized lambs increase preference for tannin-containing foods relative to non-parasitized lambs (Villalba et al., 2010), and that parasitized lambs eat more tannin-containing food than non-parasitized lambs (Lisonbee et al., 2009). In these two studies, carried out on lambs with moderate to low natural parasite infections, animals only displayed modest preference for a high-tannin food. In the present study, parasitic loads were much higher and preference for a tannin-containing food was also higher, suggesting a direct relationship between severity of parasitic infection and preference for a medicinal food. Lambs also titrate the amount of medicine (polyethylene glycol) they consume as a function of the amount of phytochemical (tannin) in their diet (Provenza et al., 2000). Other studies also suggest parasitized herbivores increase preference for tannins. Goats infected with endoparasites increased the percentage of tannin-containing heather in their diet relative to anthelmintic-treated goats (Osoro et al., 2007), and parasitized goats tended to selectively browse *Albizia anthelmintica* (a bitter plant) that leads to declines in fecal egg counts (Gradé et al. 2009). Sheep infected with adult populations of *H. contortus* eat more of the Mexican tannin-rich plant *Lysiloma latisiliquum* (Tzalam) than non-infected animals (Martinez Ortiz de Montellano et al., 2010).

Moderate concentrations of tannins (20-40 g/kg DM) have been linked to positive health effects such as increased wool and milk production (Barry and McNabb, 1999; Min et al., 1999), and increased nutrient absorption (Waghorn et al., 1987b). Tannin concentration in the present study was added at a higher concentration than the aforementioned amounts (80 g/kg DM), which could lead to adverse effects in ruminants (Barry and Manley, 1984). Nevertheless, our results show that Treatment sheep increased their intake of and preference for tannins when parasitic loads were high, choosing to increase their consumption of a nutritionally less desirable and potentially toxic food. Lambs in the Treatment group traded off, to a greater extent than Control lambs, consumption of a nutritious food (beet pulp) for the same food diluted with tannins and thus of lesser nutritional value. It is likely that the beneficial effects of tannins in that particular physiological context under a parasitic infection outweighed to some degree the potential negative effects of the phytochemical.

An important aspect of studying and analyzing behavior involves understanding the survival value and adaptive significance of a behavior

(Tinbergen, 1963). Behaviors aimed at sustaining homeostasis in living organisms such as self-medicative behavior can be understood as a type of adaptive plasticity that improves an individual's prospects for survival and reproduction (Singer et al., 2009). Thus, we predict herbivores increase their preference for secondary compounds such as tannins at the concentrations given in this study when it is adaptive, i.e., in the presence of parasitism, and decrease preference for the same compounds in the absence of disease due to costs induced by consuming secondary compounds (Hutchings et al., 2006; Singer et al., 2009).

In our study, lambs that experienced the medicinal effects of tannins not only increased their preference for tannins during parasitism relative to controls, but decreased their preference after all animals were drenched with antiparasitic agents. Likewise, lambs with natural gastrointestinal parasitic infections reduce preference for tannins after being treated with an antiparasitic drench (Lisonbee et al., 2009; Villalba et al., 2010). This does not imply that learning in the experienced group extinguished, simply that the need to eat the tannin food no longer existed. Sheep retain their ability to select from a variety of medicinal foods and supplements appropriate for attenuating the effects of illness inducing foods even 5 months after conditioning (Villalba et al. 2006). Thus, animals in our study likely discontinued their preference for tannins as need decreased and thus the cost of consuming a potentially toxic compound outweighed its potential benefit. Medicinal compounds such as tannins with negative postingestive effects at high amounts create a cost-benefit dichotomy in diet selection: the potential medicinal benefits of consuming the phytochemical must be balanced against the potentially toxic effects (Hutchings et al., 2006).

Sheep from Treatment and Control groups avoided the tannin-containing food throughout the preference tests, but the degree to which this avoidance was manifest depended on the presence or absence of parasitic infection, as well on the previous experience animals had with the antiparasitic effects of tannins. Thus, as in previous studies (Villalba et al., 2010), parasitized sheep do not manifest an absolute state of high preference for phytochemicals (tannins) with antiparasitic effects. Rather, animals display a “lower state of avoidance” for tannins when parasitized and when they learn (Treatment) about beneficial effects of tannins than when non-parasitized or when the association medicine-parasitism is absent (Control).

Consumption of Tannins and Indicators of Parasitism

I predicted consumption of tannins by treatment lambs would reduce the number of internal parasites. Consistent with this, lambs that consumed the tannin-containing food during conditioning had lower FEC, an indirect measure of parasitic burdens, than Control lambs. My results suggest quebracho tannin did not completely remove parasitic burdens of *H. contortus* in sheep heavily infected with L3 larvae, but they may diminish the severity of infection. This suggests quebracho tannin extract may be more usefully used to keep *H. contortus* infections at a lower level rather than as a treatment after infection has already reached clinical severity. Additionally, Athanasiadou et al. (2001) found that quebracho tannin extract did not halt the development of *H. contortus* larvae but decreased the viability of larvae with increasing concentrations of quebracho tannin extract. Niezen et al. (1998) saw a similar effect on lambs grazing forages with or without tannins. Infected lambs grazing sulla (12% tannins), even when manifesting high parasitic loads, held their infection to lower values than those of lambs grazing either lower tannin or no tannin-containing forages. Neizen et al. (1998) also demonstrated a trend of different tannin-containing plants seemingly having more effect on some gastrointestinal parasites than others, with sulla reducing the amount of *Tricostrongylus* in slaughtered lamb intestines.

In addition to FEC, we used other clinical indicators of infection such as packed cell volume (PCV) and red cell parameters. *H. contortus* infections induce reductions in hematocrit, hemoglobin, and red blood cell counts attributed to the blood loss caused by the blood sucking activities of the parasite (Mir et al., 2007). Even when no differences in PCV were found between groups of lambs, readings for Nov. 16 were numerically higher in the Treatment than in the Control group. In addition, PCV values increased toward the end of the study, consistent with a decrease in parasitic burdens due to chemotherapy.

Red blood cell distribution width and mean cell volume tended to be higher for the Control than for the Treatment group. Red cell distribution width is a calculation of the variation in the size of red blood cells. In some anemia's, the amount of variation (anisocytosis) in red blood cell size (along with variation in shape, poikilocytosis) causes an increase in this parameter (Walker et al., 1990). Mean cell volume is a measurement of the average size of red blood cells. This parameter is elevated when red blood cells are larger than normal (macrocytic) in some anemia's (Walker et al., 1990). Thus, consumption of tannins by the Treatment group reduced the incidence of some parameters for assessing anemia, relative to the Control group.

Internal parasites are one of the greatest disease problems in grazing livestock worldwide (Min and Hart, 2003; Waller, 2006), but their control is problematic due to the rise in drug-resistant organisms (Githiori et al., 2006; Jackson and Miller, 2006). Selection of medicinal plants and supplements is a novel and complementary alternative to other disease prevention practices such as chemotherapy.

Our findings suggest parasite loads declined after tannin ingestion and they support the hypothesis that sheep learned to modify consumption of a tannin-containing food when they experienced a parasite burden. Such learning process involved allowing animals to consume the medicinal tannin-containing food while experiencing a parasitic burden (Treatment). Disjunction of this association by consuming a food with tannins without a parasitic infection, and then consuming a food without tannins while parasitized did not enable Control animals to learn of the beneficial effects of tannins.

Once learning was achieved, preference for the medicinal food was a function of presence/absence of parasitism: Preferences for the tannin containing food declined when parasite burdens were eliminated by chemotherapy. This plasticity in diet selection is important as trained animals may trade off, to a greater extent than naïve animals, consumption of a nutritious food for a phytochemical-containing feed when infected, but not when infection subsides.

Management programs should be geared at enhancing the likelihood of medicine-relief association during parasitic infection. This could be achieved by exposing animals to phytochemical-containing forages with antiparasitic properties during peaks of parasitic infection such that consumption of a medicine and occurrence of the disease are in synchrony.

CONCLUSIONS

This study adds to the emerging field of self-medicative behavior. Our study is one of the first to show that tannin containing forages provide not only relief from parasitism, as previously demonstrated (Niezen et al., 1995; Min et al., 2003; Min and Hart, 2003), but may also be a viable means of diminishing parasitism through foraging behavior. Knowledge that animals, especially mammals, can learn has become common place. This knowledge comes primarily from the dynamic interplay between taste and postingestive feedback (Provenza, 1995). Postingestive feedback loops occur as an animal experiences the effect of the ingested substance (feed) and begins to form an association between that substance and its effect on the body. Self-medicative behaviors occur when sick animals learn to associate the effects of reduced illness with the substance consumed and begin to actively seek these substances when ill and subsequently avoid or seek at a much lower rate when not ill.

My study demonstrated the ability of lambs to recognize their own internal state (parasitism) and increase consumption of tannin-containing foods which provided relief from parasitic burdens. Our study also showed lambs did not posses an innate preference for tannin-containing foods prior to infection with parasites. On the contrary, lambs needed to experience the ingestion of tannins while infected to form a preference for this medicine. Moreover, lambs did not retain their preference for tannin-containing foods when the parasitic infection subsided after chemotherapy, i.e., when need for a medicine was absent. Thus, preference for the tannin-containing food was flexible and depended on the animal's physiological state.

My study also showed that tannins do not completely suppress parasitic infections. Rather, they reduce the incidence of the infection to subclinical severity, as estimated by the lower fecal egg counts observed in animals consuming tannins than in animals consuming the control feed (beet pulp).

One advantage of self-medicative behavior is animals consume the medicine-containing food as a function of need. Thus, consumption increases during sickness and decreases when sickness subsides. Offering choices between medicinal (i.e., foods containing plant secondary compounds) and nutritious foods is advantageous compared to force feeding medicinal foods because learning will persist and this will allow lambs to seek out medicinal foods without guidance from humans. Moreover, force feeding can increase intake of plant secondary compound-containing foods beyond the animal's needs affecting productivity and welfare.

Our research creates interesting possibilities for future studies of the various means by which tannins and other plant secondary compounds may affect gastrointestinal parasites and other diseases.

Ruminants are constantly exposed to potential treats in their environment whether from bloat (Villalba et al., 2009) excess toxins from foods (Villalba et al., 2006) or parasitism. Future avenues for research may involve exploring self-medicative behavior in pasturelands and rangelands, when animals are challenged by different types of diseases such as parasitic infections (endo and ectoparasites), bloat, or ingestion of toxin-containing feeds.

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APPENDICES

Appendix A

LABORATORY PROCEDURES

Fecal Egg Counts

Place 4 grams of feces in a beaker. To this beaker add 56 ml of zinc sulfate flotation solution and mix thoroughly using glass stir rod. After thoroughly mixed, place a strainer on a second beaker and pour mixture from beaker one into the strainer and allow solution to filter into second beaker*.

Next using a disposable pipette slowly withdraw fluid from second beaker*. Dispense fluid into both sides of the McMaster's slide and allow to sit at least 10 minutes before putting under the microscope at 10x magnification. Lastly, count the eggs in both chambers of the slide and multiply this final number by 50 to get eggs per gram of feces.

*Do not allow the contents of beaker to settle when transferring or removing fluid. Always keep solution thoroughly mixed to prevent biased results due to settling of beakers contents or flotation of eggs.

Packed Cell Volume

Blood samples where collected from each animal by jugular vein puncture into separate vacuum anticoagulant tubes by the university veterinarian. In the laboratory each sample was individually drawn into its own capillary tube and the end capped with wax. All samples were spun in the centrifuge for 2 minutes and allowed to come to rest.

Samples were analyzed by measuring distance from the start of the plasma to the end of the blood. Next just the plasma was measured and this was divided by the distance of the plasma plus blood to determine percent of blood that was plasma and percent cells.

Appendix B

SAS Output

Exposure to Beet Pulp

Least Squares Means							
Effect	Group	Day	Estimate	Standard Error	DF	t Value	Pr > |t|
Group	1		719.80	39.2771	98	18.33	<.0001
Group	2		881.13	39.2771	98	22.43	<.0001
Day		1	219.50	62.1025	98	3.53	0.0006
Day		2	967.50	62.1025	98	15.58	<.0001
Day		3	721.45	62.1025	98	11.62	<.0001
Day		4	1048.50	62.1025	98	16.88	<.0001
Day		5	1045.36	62.1025	98	16.83	<.0001
Group*Day	1	1	166.27	87.8263	98	1.89	0.0613
Group*Day	1	2	783.09	87.8263	98	8.92	<.0001
Group*Day	1	3	692.00	87.8263	98	7.88	<.0001
Group*Day	1	4	960.27	87.8263	98	10.93	<.0001
Group*Day	1	5	997.36	87.8263	98	11.36	<.0001
Group*Day	2	1	272.73	87.8263	98	3.11	0.0025
Group*Day	2	2	1151.91	87.8263	98	13.12	<.0001
Group*Day	2	3	750.91	87.8263	98	8.55	<.0001
Group*Day	2	4	1136.73	87.8263	98	12.94	<.0001
Group*Day	2	5	1093.36	87.8263	98	12.45	<.0001

Exposure to Tannin

Least Squares Means							
Effect	Group	Day	Estimate	Standard Error	DF	t Value	Pr > |t|
Group	1		1003.89	28.5485	98	35.16	<.0001
Group	2		1173.22	28.5485	98	41.10	<.0001
Day		1	842.09	45.1391	98	18.66	<.0001
Day		2	972.18	45.1391	98	21.54	<.0001
Day		3	1192.36	45.1391	98	26.42	<.0001
Day		4	1420.50	45.1391	98	31.47	<.0001
Day		5	1015.64	45.1391	98	22.50	<.0001
Group*Day	1	1	764.91	63.8363	98	11.98	<.0001
Group*Day	1	2	899.55	63.8363	98	14.09	<.0001
Group*Day	1	3	1072.36	63.8363	98	16.80	<.0001
Group*Day	1	4	1322.09	63.8363	98	20.71	<.0001
Group*Day	1	5	960.55	63.8363	98	15.05	<.0001
Group*Day	2	1	919.27	63.8363	98	14.40	<.0001
Group*Day	2	2	1044.82	63.8363	98	16.37	<.0001
Group*Day	2	3	1312.36	63.8363	98	20.56	<.0001
Group*Day	2	4	1518.91	63.8363	98	23.79	<.0001
Group*Day	2	5	1070.73	63.8363	98	16.77	<.0001

Intake initial choice

Least Squares Means								
Effect	Feed	Group	Day	Estimate	Standard Error	DF	t Value	Pr > |t|
Feed	beet			963.27	25.3760	20	37.96	<.0001
Feed	tannin			355.23	25.3760	20	14.00	<.0001

Least Squares Means								
Effect	Feed	Group	Day	Estimate	Standard Error	DF	t Value	Pr > |t|
Day			1	726.14	20.2158	40	35.92	<.0001
Day			2	592.36	20.2158	40	29.30	<.0001
Feed*Day	beet		1	996.14	28.5895	40	34.84	<.0001
Feed*Day	beet		2	930.41	28.5895	40	32.54	<.0001
Feed*Day	tannin		1	456.14	28.5895	40	15.95	<.0001
Feed*Day	tannin		2	254.32	28.5895	40	8.90	<.0001

Initial Preference

Least Squares Means							
Effect	Group	Day	Estimate	Standard Error	DF	t Value	Pr > |t|
Group	1		26.6795	2.5463	20	10.48	<.0001
Group	2		25.0005	2.5463	20	9.82	<.0001
Day		1	30.7950	2.0642	20	14.92	<.0001
Day		2	20.8849	2.0642	20	10.12	<.0001
Group*Day	1	1	32.9995	2.9192	20	11.30	<.0001
Group*Day	1	2	20.3594	2.9192	20	6.97	<.0001
Group*Day	2	1	28.5905	2.9192	20	9.79	<.0001
Group*Day	2	2	21.4104	2.9192	20	7.33	<.0001

Conditioning Beet Pulp and Tannins

Least Squares Means							
Effect	Group	Day	Estimate	Standard Error	DF	t Value	Pr > |t|
Group	control		1298.22	14.4767	378	89.68	<.0001
Group	treatment		861.86	14.4767	378	59.53	<.0001

Intake during choice 1st hour choice trials

Least Squares Means								
Effect	Feed	Group	Day	Estimate	Standard Error	DF	t Value	Pr > |t|
Feed	beet			519.78	14.3481	20	36.23	<.0001
Feed	tannin			62.0303	14.3481	20	4.32	0.0003
Group*Feed*Day	beet	1	1	657.18	35.6960	440	18.41	<.0001
Group*Feed*Day	beet	1	2	664.45	35.6960	440	18.61	<.0001
Group*Feed*Day	beet	1	3	348.27	35.6960	440	9.76	<.0001
Group*Feed*Day	beet	1	4	892.73	35.6960	440	25.01	<.0001
Group*Feed*Day	beet	1	5	102.91	35.6960	440	2.88	0.0041
Group*Feed*Day	beet	1	6	654.73	35.6960	440	18.34	<.0001
Group*Feed*Day	beet	1	7	329.82	35.6960	440	9.24	<.0001
Group*Feed*Day	beet	1	8	472.55	35.6960	440	13.24	<.0001
Group*Feed*Day	beet	1	9	570.45	35.6960	440	15.98	<.0001
Group*Feed*Day	beet	1	10	450.73	35.6960	440	12.63	<.0001
Group*Feed*Day	beet	1	11	642.27	35.6960	440	17.99	<.0001
Group*Feed*Day	beet	1	12	533.00	35.6960	440	14.93	<.0001
Group*Feed*Day	tannin	1	1	70.9091	35.6960	440	1.99	0.0476
Group*Feed*Day	tannin	1	2	70.6364	35.6960	440	1.98	0.0485
Group*Feed*Day	tannin	1	3	66.3636	35.6960	440	1.86	0.0637
Group*Feed*Day	tannin	1	4	197.36	35.6960	440	5.53	<.0001

Least Squares Means								
Effect	Feed	Group	Day	Estimate	Standard Error	DF	t Value	Pr > |t|
Group*Feed*Day	tannin	1	5	73.3636	35.6960	440	2.06	0.0404
Group*Feed*Day	tannin	1	6	145.36	35.6960	440	4.07	<.0001
Group*Feed*Day	tannin	1	7	111.91	35.6960	440	3.14	0.0018
Group*Feed*Day	tannin	1	8	127.55	35.6960	440	3.57	0.0004
Group*Feed*Day	tannin	1	9	33.4545	35.6960	440	0.94	0.3492
Group*Feed*Day	tannin	1	10	33.1818	35.6960	440	0.93	0.3531
Group*Feed*Day	tannin	1	11	10.2727	35.6960	440	0.29	0.7736
Group*Feed*Day	tannin	1	12	2.1818	35.6960	440	0.06	0.9513
Group*Feed*Day	beet	2	1	494.91	35.6960	440	13.86	<.0001
Group*Feed*Day	beet	2	2	559.73	35.6960	440	15.68	<.0001
Group*Feed*Day	beet	2	3	351.55	35.6960	440	9.85	<.0001
Group*Feed*Day	beet	2	4	852.00	35.6960	440	23.87	<.0001
Group*Feed*Day	beet	2	5	322.18	35.6960	440	9.03	<.0001
Group*Feed*Day	beet	2	6	545.36	35.6960	440	15.28	<.0001
Group*Feed*Day	beet	2	7	430.55	35.6960	440	12.06	<.0001
Group*Feed*Day	beet	2	8	515.27	35.6960	440	14.44	<.0001
Group*Feed*Day	beet	2	9	526.27	35.6960	440	14.74	<.0001
Group*Feed*Day	beet	2	10	432.00	35.6960	440	12.10	<.0001
Group*Feed*Day	beet	2	11	612.00	35.6960	440	17.14	<.0001
Group*Feed*Day	beet	2	12	513.91	35.6960	440	14.40	<.0001
Group*Feed*Day	tannin	2	1	112.91	35.6960	440	3.16	0.0017
Group*Feed*Day	tannin	2	2	137.82	35.6960	440	3.86	0.0001
Group*Feed*Day	tannin	2	3	71.1818	35.6960	440	1.99	0.0468
Group*Feed*Day	tannin	2	4	64.0909	35.6960	440	1.80	0.0733
Group*Feed*Day	tannin	2	5	11.0000	35.6960	440	0.31	0.7581
Group*Feed*Day	tannin	2	6	67.2727	35.6960	440	1.88	0.0601
Group*Feed*Day	tannin	2	7	32.5455	35.6960	440	0.91	0.3624

Least Squares Means								
Effect	Feed	Group	Day	Estimate	Standard Error	DF	t Value	Pr > |t|
Group*Feed*Day	tannin	2	8	18.8182	35.6960	440	0.53	0.5983
Group*Feed*Day	tannin	2	9	3.0909	35.6960	440	0.09	0.9310
Group*Feed*Day	tannin	2	10	20.1818	35.6960	440	0.57	0.5721
Group*Feed*Day	tannin	2	11	2.3636	35.6960	440	0.07	0.9472
Group*Feed*Day	tannin	2	12	4.9091	35.6960	440	0.14	0.8907

Intake during choice trail 7hr

Least Squares Means								
Effect	Feed	Group	Day	Estimate	Standard Error	DF	t Value	Pr > |t|
Group		1		630.87	29.6808	20	21.26	<.0001
Group		2		694.26	29.6808	20	23.39	<.0001
Feed	beet			1205.37	29.6808	20	40.61	<.0001
Feed	tannin			119.76	29.6808	20	4.03	0.0006
Group*Feed	beet	1		1119.32	41.9750	20	26.67	<.0001
Group*Feed	tannin	1		142.42	41.9750	20	3.39	0.0029
Group*Feed	beet	2		1291.42	41.9750	20	30.77	<.0001
Group*Feed	tannin	2		97.0985	41.9750	20	2.31	0.0315
Group*Feed*Day	beet	1	1	1347.55	63.2753	440	21.30	<.0001
Group*Feed*Day	beet	1	2	1329.27	63.2753	440	21.01	<.0001
Group*Feed*Day	beet	1	3	894.55	63.2753	440	14.14	<.0001
Group*Feed*Day	beet	1	4	1454.64	63.2753	440	22.99	<.0001
Group*Feed*Day	beet	1	5	398.18	63.2753	440	6.29	<.0001
Group*Feed*Day	beet	1	6	1271.36	63.2753	440	20.09	<.0001
Group*Feed*Day	beet	1	7	876.64	63.2753	440	13.85	<.0001
Group*Feed*Day	beet	1	8	996.82	63.2753	440	15.75	<.0001

Least Squares Means								
Effect	Feed	Group	Day	Estimate	Standard Error	DF	t Value	Pr > |t|
Group*Feed*Day	beet	1	9	1319.73	63.2753	440	20.86	<.0001
Group*Feed*Day	beet	1	10	1039.36	63.2753	440	16.43	<.0001
Group*Feed*Day	beet	1	11	1417.91	63.2753	440	22.41	<.0001
Group*Feed*Day	beet	1	12	1085.82	63.2753	440	17.16	<.0001
Group*Feed*Day	tannin	1	1	118.73	63.2753	440	1.88	0.0613
Group*Feed*Day	tannin	1	2	141.91	63.2753	440	2.24	0.0254
Group*Feed*Day	tannin	1	3	95.0909	63.2753	440	1.50	0.1336
Group*Feed*Day	tannin	1	4	292.64	63.2753	440	4.62	<.0001
Group*Feed*Day	tannin	1	5	206.45	63.2753	440	3.26	0.0012
Group*Feed*Day	tannin	1	6	230.00	63.2753	440	3.63	0.0003
Group*Feed*Day	tannin	1	7	228.18	63.2753	440	3.61	0.0003
Group*Feed*Day	tannin	1	8	168.27	63.2753	440	2.66	0.0081
Group*Feed*Day	tannin	1	9	72.6364	63.2753	440	1.15	0.2516
Group*Feed*Day	tannin	1	10	84.6364	63.2753	440	1.34	0.1817
Group*Feed*Day	tannin	1	11	46.2727	63.2753	440	0.73	0.4650
Group*Feed*Day	tannin	1	12	24.2727	63.2753	440	0.38	0.7015
Group*Feed*Day	beet	2	1	1215.36	63.2753	440	19.21	<.0001
Group*Feed*Day	beet	2	2	1317.00	63.2753	440	20.81	<.0001
Group*Feed*Day	beet	2	3	929.09	63.2753	440	14.68	<.0001
Group*Feed*Day	beet	2	4	1684.82	63.2753	440	26.63	<.0001
Group*Feed*Day	beet	2	5	1031.91	63.2753	440	16.31	<.0001
Group*Feed*Day	beet	2	6	1370.18	63.2753	440	21.65	<.0001
Group*Feed*Day	beet	2	7	1226.91	63.2753	440	19.39	<.0001
Group*Feed*Day	beet	2	8	1319.36	63.2753	440	20.85	<.0001
Group*Feed*Day	beet	2	9	1403.27	63.2753	440	22.18	<.0001
Group*Feed*Day	beet	2	10	1216.64	63.2753	440	19.23	<.0001
Group*Feed*Day	beet	2	11	1551.36	63.2753	440	24.52	<.0001

Least Squares Means								
Effect	Feed	Group	Day	Estimate	Standard Error	DF	t Value	Pr > |t|
Group*Feed*Day	beet	2	12	1231.18	63.2753	440	19.46	<.0001
Group*Feed*Day	tannin	2	1	220.00	63.2753	440	3.48	0.0006
Group*Feed*Day	tannin	2	2	210.18	63.2753	440	3.32	0.0010
Group*Feed*Day	tannin	2	3	125.45	63.2753	440	1.98	0.0480
Group*Feed*Day	tannin	2	4	91.5455	63.2753	440	1.45	0.1487
Group*Feed*Day	tannin	2	5	76.0000	63.2753	440	1.20	0.2304
Group*Feed*Day	tannin	2	6	135.82	63.2753	440	2.15	0.0324
Group*Feed*Day	tannin	2	7	121.36	63.2753	440	1.92	0.0558
Group*Feed*Day	tannin	2	8	42.8182	63.2753	440	0.68	0.4990
Group*Feed*Day	tannin	2	9	31.5455	63.2753	440	0.50	0.6184
Group*Feed*Day	tannin	2	10	53.6364	63.2753	440	0.85	0.3971
Group*Feed*Day	tannin	2	11	32.5455	63.2753	440	0.51	0.6073
Group*Feed*Day	tannin	2	12	24.2727	63.2753	440	0.38	0.7015

Preference choice during 1st hour

Least Squares Means							
Effect	Group	Day	Estimate	Standard Error	DF	t Value	Pr > |t|
Group	1		13.7916	1.5655	20	8.81	<.0001
Group	2		8.2573	1.5655	20	5.27	<.0001

Preference during choice trial 7hr

Least Squares Means							
Effect	Group	Day	Estimate	Standard Error	DF	t Value	Pr > |t|
Group	1		13.4359	1.5038	20	8.93	<.0001
Group	2		7.3020	1.5038	20	4.86	<.0001

Intake by period after drench 1st hour

Least Squares Means								
Effect	Feed	Group	Day	Estimate	Standard Error	DF	t Value	Pr > |t|
Feed	beet			580.09	20.5616	20	28.21	<.0001
Feed	tannin			49.5130	20.5616	20	2.41	0.0258
Group*Feed	beet	1		542.90	29.0784	20	18.67	<.0001
Group*Feed	tannin	1		68.6364	29.0784	20	2.36	0.0285
Group*Feed	beet	2		617.29	29.0784	20	21.23	<.0001
Group*Feed	tannin	2		30.3896	29.0784	20	1.05	0.3084
Group*Feed*Day	beet	1	3	562.64	42.0384	240	13.38	<.0001
Group*Feed*Day	beet	1	4	614.18	42.0384	240	14.61	<.0001
Group*Feed*Day	beet	1	5	560.09	42.0384	240	13.32	<.0001
Group*Feed*Day	beet	1	6	414.00	42.0384	240	9.85	<.0001
Group*Feed*Day	beet	1	7	504.55	42.0384	240	12.00	<.0001
Group*Feed*Day	tannin	1	1	47.6364	42.0384	240	1.13	0.2583
Group*Feed*Day	tannin	1	2	66.9091	42.0384	240	1.59	0.1128
Group*Feed*Day	tannin	1	3	108.55	42.0384	240	2.58	0.0104
Group*Feed*Day	tannin	1	4	73.3636	42.0384	240	1.75	0.0822
Group*Feed*Day	tannin	1	5	137.36	42.0384	240	3.27	0.0012
Group*Feed*Day	tannin	1	6	9.0000	42.0384	240	0.21	0.8307
Group*Feed*Day	tannin	1	7	37.6364	42.0384	240	0.90	0.3715
Group*Feed*Day	beet	2	1	605.73	42.0384	240	14.41	<.0001

Least Squares Means								
Effect	Feed	Group	Day	Estimate	Standard Error	DF	t Value	Pr > |t|
Group*Feed*Day	beet	2	2	697.91	42.0384	240	16.60	<.0001
Group*Feed*Day	beet	2	3	883.36	42.0384	240	21.01	<.0001
Group*Feed*Day	beet	2	4	631.73	42.0384	240	15.03	<.0001
Group*Feed*Day	beet	2	5	562.09	42.0384	240	13.37	<.0001
Group*Feed*Day	beet	2	6	438.36	42.0384	240	10.43	<.0001
Group*Feed*Day	beet	2	7	501.82	42.0384	240	11.94	<.0001
Group*Feed*Day	tannin	2	1	36.0909	42.0384	240	0.86	0.3915
Group*Feed*Day	tannin	2	2	53.7273	42.0384	240	1.28	0.2025
Group*Feed*Day	tannin	2	3	8.2727	42.0384	240	0.20	0.8442
Group*Feed*Day	tannin	2	4	11.2727	42.0384	240	0.27	0.7888
Group*Feed*Day	tannin	2	5	76.9091	42.0384	240	1.83	0.0686
Group*Feed*Day	tannin	2	6	23.1818	42.0384	240	0.55	0.5818
Group*Feed*Day	tannin	2	7	3.2727	42.0384	240	0.08	0.9380

Intake by period after drench 7hr

Least Squares Means								
Effect	Feed	Group	Day	Estimate	Standard Error	DF	t Value	Pr > |t|
Feed	beet			1344.90	38.7978	20	34.66	<.0001
Feed	tannin			85.2597	38.7978	20	2.20	0.0399

Intake comparison 1st hour during infection and after drench

Least Squares Means									
Effect	Feed	Phase	Group	Day	Estimate	Standard Error	DF	t Value	Pr > |t|
Feed	beet				548.22	17.1000	20	32.06	<.0001

Least Squares Means									
Effect	Feed	Phase	Group	Day	Estimate	Standard Error	DF	t Value	Pr > |t|
Feed	tannin				46.6526	17.1000	20	2.73	0.0130
Group*Feed	beet		1		532.42	24.1830	20	22.02	<.0001
Group*Feed	tannin		1		67.4545	24.1830	20	2.79	0.0113
Group*Feed	beet		2		564.03	24.1830	20	23.32	<.0001
Group*Feed	tannin		2		25.8506	24.1830	20	1.07	0.2978
Group*Feed*Phase	beet	After	1		542.90	26.7781	20	20.27	<.0001
Group*Feed*Phase	beet	During	1		521.94	26.7781	20	19.49	<.0001
Group*Feed*Phase	tannin	After	1		68.6364	26.7781	20	2.56	0.0185
Group*Feed*Phase	tannin	During	1		66.2727	26.7781	20	2.47	0.0224
Group*Feed*Phase	beet	After	2		617.29	26.7781	20	23.05	<.0001
Group*Feed*Phase	beet	During	2		510.77	26.7781	20	19.07	<.0001
Group*Feed*Phase	tannin	After	2		30.3896	26.7781	20	1.13	0.2698
Group*Feed*Phase	tannin	During	2		21.3117	26.7781	20	0.80	0.4355

Intake comparison 7hr during and after drench

Least Squares Means									
Effect	Feed	Phase	Group	Day	Estimate	Standard Error	DF	t Value	Pr > |t|
Feed	beet				1291.26	33.7287	20	38.28	<.0001
Feed	tannin				88.9253	33.7287	20	2.64	0.0158

Preference comparison 1st hour during infection and after drench

Least Squares Means								
Effect	Phase	Group	Day	Estimate	Standard Error	DF	t Value	Pr > |t|
Group		1		8.2593	1.3920	20	5.93	<.0001
Group		2		4.8291	1.3920	20	3.47	0.0024

Preference comparison 7hr during infection and after drench

Least Squares Means								
Effect	Phase	Group	Day	Estimate	Standard Error	DF	t Value	Pr > |t|
Group		1		8.5750	1.2134	20	7.07	<.0001
Group		2		5.0339	1.2134	20	4.15	0.0005

Fecal Egg Counts

Least Squares Means							
Effect	Group	Period	Estimate	Standard Error	DF	t Value	Pr > |t|
Group*Period	1	4	1777.27	639.40	160	2.78	0.0061

Least Squares Means							
Effect	Group	Period	Estimate	Standard Error	DF	t Value	Pr > |t|
Group*Period	1	6	3050.00	639.40	160	4.77	<.0001
Group*Period	1	7	3563.64	639.40	160	5.57	<.0001
Group*Period	2	4	4572.73	639.40	160	7.15	<.0001
Group*Period	2	6	4481.82	639.40	160	7.01	<.0001
Group*Period	2	7	5309.09	639.40	160	8.30	<.0001

Packed Cell Volume

Least Squares Means							
Effect	Group	Period	Estimate	Standard Error	DF	t Value	Pr > |t|
Period		3	42.5455	1.8685	100	22.77	<.0001
Period		5	37.1364	1.8685	100	19.88	<.0001
Period		6	40.5000	1.8685	100	21.68	<.0001
Period		7	41.9545	1.8685	100	22.45	<.0001
Period		8	37.6818	1.8685	100	20.17	<.0001
Period		9	51.6818	1.8685	100	27.66	<.0001